

The Effect of Glyphosate And Ciprofloxacin Exposure On The Gut Bacterial Microbiota Diversity of *Rhinella Arenarum* (Anura: Bufonidae) Tadpoles

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Abstract

The high load of agrochemicals and antibiotics coexisting in aquatic environments within agroecosystems represents a risk for wildlife. As the gut microbiota plays a key role on its host's functioning and is sensitive to a wide variety of pollutants, its study allows evaluating organisms' health and therefore, the ecosystem. We studied toxic effects of commercial formulations of a glyphosate-based herbicide (GBH) and the antibiotic ciprofloxacin (CIP) on gut bacterial microbiota diversity of the common toad (*Rhinella arenarum*) tadpoles, considered a sentinel species. The study was carried out by classic microbiological analysis and matrix-assisted laser desorption ionization-time of flight mass spectrometry. The microbiota from GBH treatment had greater taxa diversity and richness, including some genera, such as *Proteus* spp. and *Yersinia* spp. that were absent in control. In contrast, microbiota from CIP treatment registered a decrease of diversity indexes, dominance of *Aeromonas* spp. and presence of *Leclercia* spp. The GBH-CIP treatment showed changes in taxa composition, including decrease of *Klebsiella* spp. and *Pseudomonas* spp. and trends of individual pollutant treatments. For all cases, changes in the composition of bacterial community (dysbiosis) were linked to a significant decrease in tadpoles' weight. To the best of our knowledge, this is the first report on the disruption of gut bacterial microbiota of tadpoles by a mixture of two contaminants of emerging concern worldwide. These findings contribute to understanding how the presence of two co-occurring pollutants in freshwaters results in deleterious effects on the amphibian community and potentially affect the microbiota of those environments.

Introduction

Classic studies based on gut contents have classified tadpoles as herbivores, detritivores, microphagous, and suspension feeders (Lajmanovich 1997, 2000). For these reasons, tadpoles transfer nutrients, and energy between aquatic and terrestrial ecosystems (Capps et al. 2015). Those herbivorous tadpoles' species that mostly obtain significant nutrition from autotrophic sources are likely to be assisted by the gut microbiota (Altig et al. 2007). In this sense, Lajmanovich et al. (2001) carried out a quantitative description of all gut bacteria of *Rhinella arenarum* tadpoles and defined them as reservoirs of bacteria of sanitary interest, indicators of contamination and health risk in aquatic environments.

At present, where the load of contaminant of emerging concern (CECs) (e.g. pharmaceuticals and pesticides) is increasing worldwide in water bodies every day as a result of intensive agriculture and livestock practices (Varol and Sünbül 2017), it is crucial to evaluate their effect on the bacterial community in freshwaters and organisms (Evariste et al. 2019). The stability of microbial community structure is important not only for the health of the host organisms but also for the overall functioning of freshwater ecosystems due to its primary productivity (Vera et al. 2010; Villeneuve et al. 2011; Lozano et al. 2020). Amphibians have a complex life cycle and the species-specific physiologic and behavioural approaches can be used as a reliable model for evaluation of biological toxic effects of environmental pollutants (Hopkins 2007). Exposure to herbicides during the developmental stages of tadpoles can induce immediate and lasting alterations to gut microbiome (Shehata et al. 2013; Knutie et al. 2018). Changes in the composition of these bacterial communities (called dysbiosis) could lead to a disruption

of metabolic capacities, increase susceptibility to disease, pathogenic infections and lead to increased risk of amphibian decline (Jiménez and Sommer 2016). Furthermore, the occurrence of pharmaceutical residues (e.g. antibiotics) as CECs in the aquatic environment alarm scientific community in the last decades (Milić et al. 2013, Godoy and Sánchez 2020). The presence of antibiotics in aquatic systems is an issue of heightened interest throughout the world, due to bacterial acquisition of resistance to antibiotics (Kurenbach et al. 2018). Several studies have revealed that antibiotics can affect the gut bacteria diversity (e.g. Buffie et al. 2012, Taur et al. 2012, Peltzer et al., 2017), and increase the risk of bacterial infections in frogs (Weng et al. 2017).

Glyphosate (GLY) is the herbicide more used worldwide (Benbrook 2016). In aquatic environment, a concentration of 3.49 mg/L has been estimated in the worst-case scenario (Wagner et al. 2013). GLY performs as an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), not only in crop plants but also in bacteria. Microbial communities have been studied to be affected by GLY and other herbicide cocktails (Lozano et al. 2020). Inhibiting results on EPSP synthase from gut microbiota has been reported, affecting principally beneficial bacteria. Consequently, researchers have suggested that GLY can cause gut dysbiosis, a disturbance that is characterized by an imbalance between beneficial and pathogenic microorganisms (Rueda-Ruzafa et al. 2019). Indeed, skin bacterial communities from tadpoles of cricket frog (*Acris blanchardi*) shown to be affected by exposure to 2.5 mg/L of the commercial formulation of GLY (Krynak et al. 2017). However, additional studies on the effects of pesticide exposure on amphibian microbiome are needed (McCoy et al. 2018).

At the same time, fluoroquinolones are one of the main frequently detected types of antibiotics in the aquatic environments (Sukul and Spiteller 2007), and ciprofloxacin (CIP) was described as the most widely prescribed fluoroquinolone in the world (Picó and Andreu 2007). Besides, CIP is used at a great scale in veterinary medicine for livestock (Githinji et al. 2011). A recent study has reported concentrations of CIP \approx 16 μ g/L in several wastewaters (Danner et al. 2019) with higher values up to 150 μ g/L in the worst-case scenario of residual effluents (Martins et al. 2008). CIP is a potent activity antibiotic against gram-negative (GN) and gram-positive (GP) bacteria. It interferes in DNA synthesis by inhibiting DNA gyrase (Córdova-Kreylos and Scow 2007). CIP is a typical model antibiotic in studies of human gut microbiome disruption (Dethlefsen and Relman 2011). Furthermore, Schlomann et al. (2019) examined microbial dynamics in zebrafish (*Danio rerio*) gut microbiota culture, and they demonstrated that sublethal CIP doses caused severe drops in bacterial abundance. Likewise, Peltzer et al. (2017) observed emaciated tadpoles with severe feces deposition after exposition to CIP.

It is known that due to their large-scale use, GBHs and CIP are present in surface waters matrices worldwide (Reinstorf et al. 2008, Jayasumana et al. 2015, Gomes et al. 2019). In crop-livestock systems where pesticides are applied in crops at the time veterinary drugs are supplied to livestock, GBH and CIP are likely to be found together, and their accumulation on food crops represents a direct pathway for their insertion into the food chain and health risk (Gomes et al. 2020). The accumulation of those substances in food crops represents. Interactive effects of GBH and CIP were observed in water used for crops irrigation (Gomes et al. 2019). Recently, our laboratory analysed the combined effects of GBH and the

antibiotic CIP at environmental relevant exposures (Cuzziol Boccioni et al. 2020). This was the first study to investigate how these two pollutants affect the health of *R. arenarum* tadpoles, causing morphological abnormalities, thyroid disruption, and delayed development. However, the effect of those pollutants on tadpoles' gut microbiota communities remains unstudied. Considering the importance of gut microbiota to amphibian tadpoles, common toad (*R. arenarum*) tadpoles were used to study the toxic effects of GBH and CIP exposures on community diversity and structure of gut bacterial microbiota of tadpoles by classic microbiological analysis and to identify the microbial species by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The effect of pollutants on the gut microbiota will help to elucidate the comprehensive impact of environmental relevant exposure to GBH and CIP in pond environments in which tadpoles are raised, on the amphibian community with implications of wildlife conservation.

Materials And Methods

2.1. Chemicals

Bioassays solutions were prepared using a commercial formulation of a GBH (74.7 % active ingredient, *N*-phosphonomethyl glycine and inert adjuvant *quantum satis*; Roundup Ultra-Max®, Monsanto® Argentina;), and CIP (Sigma–Aldrich, Germany). Ultra-Max® GBH formulation and Sigma-Aldrich CIP were analytically evaluated in previous works, and for both cases, the error did not exceed 5% of the nominal concentrations (Lajmanovich et al. 2019; Peltzer et al. 2017). The chemicals were tested individually in nominal concentrations considered as relevant in worst-case scenarios for natural lentic aquatic systems (Cuzziol Boccioni et al. 2020): GBH = 2,5 mg L⁻¹ and CIP = 100 µg. L⁻¹, and in 50:50% v/v of individual concentrations. The solutions were prepared with dechlorinated water (DW), which was also used as a negative control (CO). The solutions were totally renewed every 48 h.

2.2. Organisms and experimental design

Tadpoles of the South American toad *R. arenarum* were used in this study, as it is considered a sentinel species and has been extensively used in local ecotoxicological researches (e.g. Bionda et al. 2011, Hutler Wolkowicz et al. 2012, Lajmanovich et al. 2019). Egg strings of toads were collected in temporary ponds of the natural alluvial plain of the Paraná River (31 ° 39 '45 "S, 60 ° 34 '36" W) considered an unpolluted site (Lajmanovich et al. 2019, Cuzziol Boccioni et al. 2020) and they were transported in dechlorinated water (DW) to the laboratory.

For the bioassay, 10 embryos (Gosner Stage, EG 3–4) were added to each glass flask with 200 ml of treatment solution. When individuals reached EG 23–24, they began being treated with 1000 ml of solution and fed with rations of approximately 0.2 g of organic lettuce per treatment every 48 h. The assay was carried out in triplicate (meaning three flasks with 10 organisms, total n = 30 for each treatment), under experimental conditions according to Cuzziol Boccioni et al. (2020). The assay finished after 14 days, when approximately 90% of exposed and CO tadpoles reached GS 26 (beginning of premetamorphosis).

The experiments followed the regulations of the American Society of Ichthyologists and Herpetologists (ASIH 2004). As the study of the gut microbiota required the isolation of fresh intestinal content, the individuals had to be sacrificed and immediately processed for morphological and microbiological analysis. Individuals were sacrificed by immersion in a solution of 0.1% tricainemethanesulfonate (TMS, MS-222) buffered at pH 7.8 with NaHCO₃, following the protocol of the Animal Euthanasia Guide proposed by the Institutional Committee for the Care and Use of Animals (IACUC), and that of the bioethics committee of the FBCB-UNL (Res. N° 388/06).

2.3. Morphological parameters

At the end of the bioassay, 15 individuals from each treatment (5 from each replica) were randomly selected for morphological evaluation. The organisms were fixed in 10% Formaldehyde and preserved in 70% alcohol. The individuals were dry weighed (W, in mg) on a Pioneer Ohasus® digital scale with 0.0001 g precision, and evaluated under an Arcano® stereoscopic magnifying glass with a Moticom® digital camera attached, to determine the total length (TL, in mm) and the stage of development (GS) according to Gosner (1960)

2.4. Microbiota sampling and analysis

Intestinal tracts of 15 individuals per treatment were aseptically removed, weighted and pooled in 3 samples (one from each replica, consisting of 5 individuals each) due to the low tissue volumes. Pool samples were homogenized in 500 µL of sterile peptone water using sterile glass beads (425–600 µm diameter) for intestinal walls rupture. Serial dilutions (up to 1/10000) of homogenized intestines were plated onto nutrient agar plates (0.5% pluripeptone; 0.3% meat extract; 0.8% NaCl) and incubated at 37°C for 24 h. The plate count method was used to calculate the amount of colony colony-forming units per gram of intestine (CFU/g) for evaluating quantitative differences between treatments.

In order to study diversity of species, 20 CFUs were randomly selected from the plates of each pool sample (Total N per treatment = 60 CFUs). Each isolated CFUs was individually re-suspended in 1 mL nutrient broth medium (0.5% pluripeptone; 0.3% meat extract; 0.8% NaCl, agar 2%) and incubated at 35–37 ° C overnight. Each CFU was first morphologically characterized by gram staining reaction and seven biochemical tests: triple sugar iron agar (TSI), citrate, indole, motility, urease, fenilalanine and lysine-iron. For the strains that it was possible, the identification was made using phenotypic profiling (according to Hawkey 2006, Lopardo et al. 2016, Ochoa and Ochoa 2017), but the unusual and difficult-to-identify strains required further analysis. Those strains were identified by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) using the VITEK MS system (bioMérieux). MALDI-TOF based identification was also used to confirm the identification of 10% of the strains identified by classical biochemical tests.

2.5. Data analysis

All data regarding morphological biomarkers are reported as the mean ± SD. Effects of treatments on TL and W were analyzed with ANOVA followed by post-hoc Dunnett's Comparison Test. Differences in GS

and CFU/g of tissue were evaluated by Kruskal-Wallis test followed by Dunn's post hoc test. Plate count results are expressed as CFU and were used to calculate CFU/g of tissue (gut) as follow:

$$[\text{N}^\circ \text{ colonies/ml plated} * \text{DF}] / [\text{grams tissue/ml original homogenate}] = \text{N}^\circ \text{ colonies/gram tissue} = \text{CFU/g tissue.}$$
 Data is shown as log (CFU/g of intestine).

Alpha diversity parameters (Chao1 richness estimator, Dominance index, Shannon and Simpson diversity index) were calculated using PAST 3.22 Software (Hammer et al. 2001), and are expressed as the mean \pm SD (from replicas). Taxa richness is expressed by total genera per treatment. MANOVA (Wilks' lambda multivariate test statistic) was performed to determine whether there were significant overall differences in microbiotic diversity parameters among treatments, and subsequent univariate analysis of variance (ANOVA) test followed by Dunnett's post hoc-tests were done for each parameter. Beta diversity analysis was performed to investigate the structural variation of bacteria communities across treatments and visualized via principal components analysis (PCA) and unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering (adapted from Jiang et al. 2013 and Ya et al. 2019).

For statistical analysis, data distribution was assessed for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test). BioEstat software 5.0 (Ayres et al. 2008) and InfoStat software (Di Rienzo et al. 2015) were used for statistical analysis, and significance was accepted as $p < 0.05$.

Results

3.1. Morphological parameters

The mean value of CO tadpoles' weight at the end of the assay was 69.98 ± 0.87 mg. Tadpoles treated with GBH, CIP and GBH-CIP showed a significant decrease (between 13–9%) on weight respect to CO ($F = 5.8$, $p < 0.01$; Fig. 1a). Mean weight in tadpoles from GBH treatment was 61.06 ± 0.62 mg, while in CIP was 60.06 ± 0.85 mg, and in GBH-CIP treatment, 63.78 ± 0.97 mg. The mean value of CO tadpoles' length was 9.36 ± 0.56 mm, while the values from treatments (9.58 ± 0.73 , 9.8 ± 0.75 , and 9.71 ± 0.42 mm for GBH, CIP and GBH-CIP, respectively) did not result in significant differences ($F = 2.6$, $p > 0.05$; Fig. 1b). On average, tadpoles from CO and all treatments excepted for GBH, ended the assay in a mean GS 27, while those from GBH showed lower GS (mean GS 26, $KW = 8.559$, $p < 0.05$, Fig. 1b).

3.2. Gut bacteria analysis

CFUs/g of tissue were significantly higher in GBH respect to CO (increase of 4%, $KW = 14.930$, $p < 0.01$; Fig. 2a). Diversity of intestinal microbiota in tadpoles exposed to GBH, CIP and GBH-CIP treatments varied significantly respect to CO (MANOVA Wilks' Lambda $\lambda = 0.0041$; $F = 4,84$; $p < 0.01$). Chao richness estimator ($F = 11.8$, $p < 0.01$), and both Shannon and Simpson diversity index ($F = 29$; $p < 0.01$) increased in microbiota communities from GBH while in CIP communities, diversity markedly decreased and dominance was significantly higher ($F = 27$, $p < 0.01$) than in CO (Table 1). In GBH-CIP treatment, diversity and richness index were similar to CO, but the relative abundance of some genus was different (Fig. 2b). *Escherichia coli*, as well as *Klebsiella* spp., *Shewanella* spp., *Aeromonas* spp. and *Pseudomonas* spp.

were identified as the predominant taxa in the CO group. After treatment, the relative prevalence of *Aeromonas* spp. increased in detriment of the other named genera. This genus was dominant in both CIP and mixed treatments. In contrast, *Aeromona* spp. dominance was lower in the GBH treatment group (Table 1). *Yersinia* spp. and *Proteus* spp. were isolated in these groups and not in the others.

Table 1 Diversity indexes of gut bacterial microbiota of *R. arenarum* tadpoles from control (CO), and treatments with a glyphosate-based herbicide (GBH) and the antibiotic ciprofloxacin (CIP).

	CO	GBH	CIP	GBH-CIP	Statistical analysis
Total_Genera Richness	9	11	7	8	
Chao-1	6.66±0.76	10.25±1.8	4.33±0.57	8±1.32	F= 11.8 *
Dominance	0.248±0.04	0.133±0.02	0.51±0.07	0.216±0.06	F= 27.4*
Simpson_1-D	0.74±0.04	0.86±0.02	0.48±0.06	0.77±0.07	F= 29.7*
Shannon_H	1.59±0.14	2.11±0.15	0.91±0.1	1.70±0.21	F= 29.48*

* $p \leq 0.0$; numbers in bold indicate significantly different from CO ($p < 0.05$ in Dunnet or Dunn post hoc test).

The PCA for Beta diversity resulted in four groups corresponding to treatments samples (Fig. 3a). Treatments showed to be scattered through components axis, indicating that microbial communities of gut samples from CO, GBH, CIP and GBH-CIP varied among taxa. In the X axis (where most of variation was represented, $\lambda_{PC1} = 59,2\%$), CO community were closely related to GBH and GBH-CIP ones, while CIP community was the most different treatment in according to the taxa present in each one (Fig. 3a). Similarly, in UPGMA the GBH-CIP treatment was closely related to CO, and while GBH was closer to them than CIP, based on the relative abundance of taxa in each treatment (Fig. 3b).

Discussion

Amphibians face anthropogenic pollution threats from different sources, and their associated gut microbiomes are highly sensitive to these pollutants (Jiménez and Sommer 2016). Pesticides are likely to co-occur with antibiotics that are worldwide used in human and veterinary medicine and arrive to aquatic environments from agroecosystems and swine, chicken and cow feedlots without any treatments (Rico et al. 2014). The interactive impact of antibiotics and herbicides on gut microbiota disturbance has rarely been studied (Zhan et al. 2018). The present study reinforced that GBH and CIP, individually and in mixture, altered gut microbiota composition of *R. arenarum* tadpoles. A decrease in weight mass and stage of development in GBH treatment- was also observed in treatments with microbiota alteration.

It is known that gut microbiota plays key roles in host vital functions as immune-system modulation, digestion, biotransformation and protection against pathogens (Sommer et al. 2013). Therefore, dysbiosis of microbiota, as well as alteration of its functional optimization can lead to severe health problems for hosts (Claus et al. 2016). In this study, decrease on tadpoles' weight from both individual and mixture pollutants treatments was observed, together with a disruption of normal microbiota community structure. Moreover, in GBH treatment, tadpoles also showed a delay on development. Similar to previous studies that described gut microbiota shifts related to weight and changes on development (Zhang et al. 2020; Chai et al. 2018), these results suggest a relation between microorganism community and the animals' physiology (Nehra et al. 2016) and enhance the importance of study gut microbiota to estimate impact of environmental chemicals on health and fitness of wildlife, environments and humans (Nguyen et al. 2015).

The taxa that have been found in this study as part of the microbial communities of *R. arenarum* tadpoles mostly belong to the bacilli Gram (-) Enterobacteriaceae family, also *Aeromonas* and *Pseudomonas* species. The genera of bacteria found in the microbial communities of CO tadpoles coincided with those described in pioneering works in the area that share the culture sowing methodology (Hird et al. 1983, Lajmanovich et al. 2001). After two weeks of exposure to GBH, microbial communities from the tadpoles' gut had significantly changed their structure. The diversity and genus richness indexes of gram-negative bacteria increased considerably, while the dominance of the taxa registered in the control (i.e. *E. coli*), decreased. GBHs have already been reported to disrupt the gut microbiota of animals likely to live near agricultural sites (i.e., bees, Motta et al. 2018; water fleas *Daphnia spp.*, Suppa et al. 2020). Besides, it was also found that GBH altered the diversity of the soil microbes (Wolmarans 2014) and can favor to certain species which perform less efficiently in the other conditions (Imparato et al. 2016). In general, it is agreed that diversity is given by the variation in environmental conditions and availability of nutrients (Goldfarb et al. 2011). In this sense, the dysbiosis observed here for the microbiota community of GBH-treated *R. arenarum* tadpoles, given by an increase in diversity and evenness of taxa, could be related to the fact that GLY positively influences the bacterial growth since some Gram (+) and Gram (-) bacteria use it as a source of carbon, nitrogen and phosphorus (Van Eerd et al. 2003). Other alterations in the normal conditions of the intestinal lumen due to xenobiotics as GLY could also contribute to the microbiota shift: it has been suggested that mucous layer of the intestine of mammals is affected by pollutants, and dietary emulsifiers are capable of altering the microbiota (Chassaing et al. 2015; Lozano et al. 2018).

Another explanation for the shift in bacteria community composition in GBH treatment may be related to the ability of some bacteria to transform GLY (Sviridov et al. 2015). Some bacteria transform GLY into aminomethylphosphonic acid (AMPA) by the enzyme glyphosate oxidoreductase, and use this metabolite or directly the GLY molecule to obtain phosphate for their metabolism by C-P bond break down (Imparato et al. 2016). Thus, it can be inferred that GBH application may induce to an artificial selection that stimulates existing bacteria capable of degrading the herbicide (Villarreal-Chiu et al. 2017). In accordance, some of the taxa increased in GBH treatment of our study, such as *Enterobacter spp.* and *Providencia spp.*, are known to have associative traits to GLY degradation or use as a substrate (Nourouzi

et al. 2011, Kryuchkova et al. 2014). From an ecological point of view, the decrease of some bacterial species could release ecological niches that would be occupied by others (Blot et al. 2019). Regarding this, the importance of deepening the study of the relationships between the different taxa that make up the intestinal microbiota here is highlighted, and the imbalance that could be generated between beneficial and pathogenic bacteria for amphibian tadpoles.

Concerning the quantification of CFU, GBH treatment showed a significant increase compared to CO. This effect could be related, among various factors, to host transcriptional changes enriched for lipid and carbon metabolism, as is suggested by Suppa et al. (2020). Moreover, studies on dynamics of bacteria communities of the soil and rhizosphere, associated the increase of fast-growing bacteria abundance with the availability of carbon compounds in GLY presence (Imparato et al. 2016). The increase in the amount of CFU in the GBH treatment probably corresponds to the increase in those taxa capable of degrading GLY. Consequently, the production of AMPA would increase, together with its potential risks to hosts animals and human health (e.g. impairment of DNA reparation and mRNA synthesis, Allemann 2019, de Brito Rodrigues et al. 2019).

On the other hand, most of the studies of the effect of antibiotics on the intestinal microbiota are focused on human health, and warn about the consequences of prolonged treatments and permanent loss of certain fundamental taxa for the maintenance of healthy gut (Jakobsson et al. 2010, Pop et al. 2016). Similarly, it is important to pay attention to the effects that drug residues may have on the bacterial communities of non-target organisms, since these are frequent in water bodies (Peltzer et al. 2017, Hu et al. 2020). In our study, CIP treatment induced dysbiosis on gut microbiota of *R. arenarum* tadpoles by reduction of taxa diversity and increase dominance of a single genus. *Aeromonas* spp. represented more than 50% of relative taxa abundance on microbial gut community, assuming its resistance to CIP. This result is consistent with other studies that reported multidrug-resistance (including CIP) of *Aeromonas* spp. from wild animals (Dias et al. 2018).

The presence of dysbiosis on CIP treated tadpole can lead to serious consequences on tadpoles and other wild animals, since *Aeromonas* spp. identified here (*A. veronii* and *A. hydrophila*) had been associated with several diseases in humans and fishes (Rahman et al. 2002, Toranzo et al. 2005, Janda and Abbot, 2010). Skwor et al. (2014) warned about the risk of emerging resistant *Aeromonas* spp. in the environment and organisms, due to the overuse of antibiotics in both human and veterinary medicine. In addition, *Leclercia* spp. was another taxon that highlight dysbiosis in CIP treatment, since it did not appear on CO nor GBH treatments. Yehia (2013) reported multiple resistance to antibiotics (including CIP) in *Leclercia* spp. strains isolated from farm poultry intestinal tracts, and enhanced the health risks remaining on inadequate use of a wide spectrum of antibiotics for different interest.

In the present study, richness and diversity index in the gut microbiota from GBH-CIP treatment were similar to CO, but the taxa composition showed to be different. Some genera from CO as *Klebsiella* spp. and *Pseudomonas* spp. were decreased or absent in the mixture treatment. Additionally, some trends observed for individual pollutant treatments were repeated in CHB-CIP: increase of *Enterobacter* spp. and

presence of *Proteus* spp. (as in GBH), and increase of *Aeromonas* spp. and presence of *Leclercia* spp. (similar to CIP treatment). It is more than clear that pressure of both xenobiotics interacts to influence microbial community structure. Results observed in GBH-CIP mixture treatment not only confirmed the susceptibility of gut bacterial microbiota in *R. arenarum* tadpoles to different type of pollutants individually, but also enhance their effects in mixtures, as they are more likely happen in the environment (Ramakrishnan et al. 2019). To the best of our knowledge, this is the first report on disruption of gut microbiota of amphibian tadpoles by a mixture of an antibiotic and herbicide. As it is clearly aimed on a recent review, CECs affect gut bacteria and have great imbalance on host health (Tsiauosiss et al. 2019). More studies are need to elucidate how real-life scenarios with complex CECs mixtures can affect tadpole microbiota and ultimately, life aquatic health.

Overall, gut bacterial microbiota demonstrated to be a key endpoint for evaluating the effects of pollutants on non-target animals as amphibians' tadpoles. Last years, there has been a growing interest and concern about its diversity and structure variation due to changes in environmental conditions and pressures in order to understand its complex symbiotic relations with hosts' life (Evariste et al. 2019), and the bacterial resistance due to exposure to antibiotics such as CIP (Jørgensen et al. 2013).

Conclusion

The results of our study suggest that commercial formulations of GBH and CIP, individually and in mixture, caused severe gut bacterial microbiota dysbiosis of *R. arenarum* tadpoles affecting individuals' weight. Apart from the already known direct effects of these pollutants on tadpoles, the dysbiosis they cause may lead to additional physiological problems through alteration of the gut microbiota normal functioning, including metabolic activities related to nutrients and energy recovery. Further studies about the gut microbiota on tadpoles exposed to pollutants mixtures such as herbicides (e.g. GLY) and antibiotics (e.g. CIP) and the potential use of microbiota composition as biomarker to apply not only to environmental risk assessment but also on wild animals health are urgent needed. Furthermore, the bacterial communities' dynamics in face of CECs is essential for understanding bacterial resistance, a highly-complex and growing concern nowadays for human health.

Declarations

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Conflicts of interest / Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material (data transparency)

Data presented in this study are available on request from the corresponding author.

Code availability (software application or custom code)

Not applicable.

Authors contributions

Conceptualization: Guillermo García Efron (GGE); Methodology: Ana P. Cuzziol Boccioni (APCB); Formal analysis and investigation, Writing - original draft preparation: APCB, Rafael C. Lajmanovich (RCL); Writing - review and editing: GGE, Paola M. Peltzer; Resources: RCL, GGE; Supervision: GGE.

Animal Research (Ethics approval)

Animals were treated according the Institutional Committee for the Care and Use of Animals (IACUC), and approval was obtained from the bioethics committee of the FBCB-UNL (Res. N° 388/06).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Plant Reproducibility

Not applicable.

Clinical Trials Registration

Not applicable.

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Figures

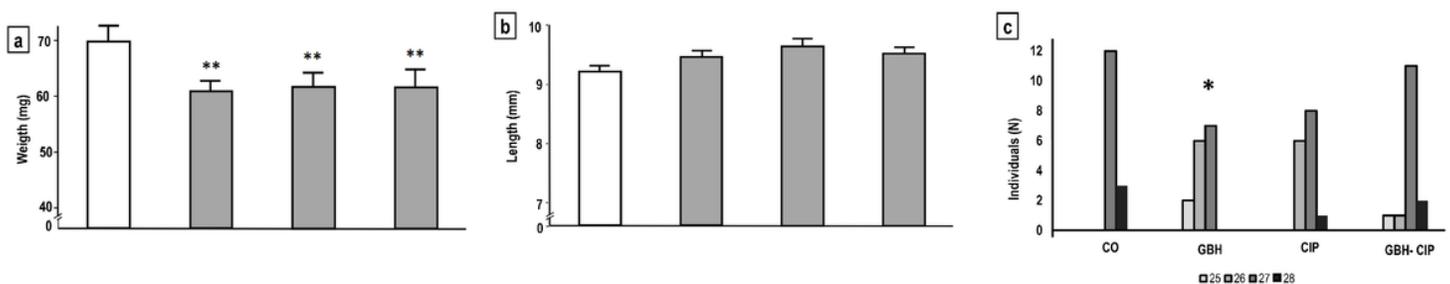


Figure 1

Morphological parameters (mean \pm SD) of tadpoles of *R. arenarum* exposed to glyphosate-based herbicide (GBH), ciprofloxacin (CIP) and their 50:50 % mixture (GBH-CIP), compared to control group (CO). a. Weight; b. Length; c. Gosner Stage. Significantly different from CO * $p < 0.05$ ** $p < 0.01$ in Dunnet or Dunn post hoc test)

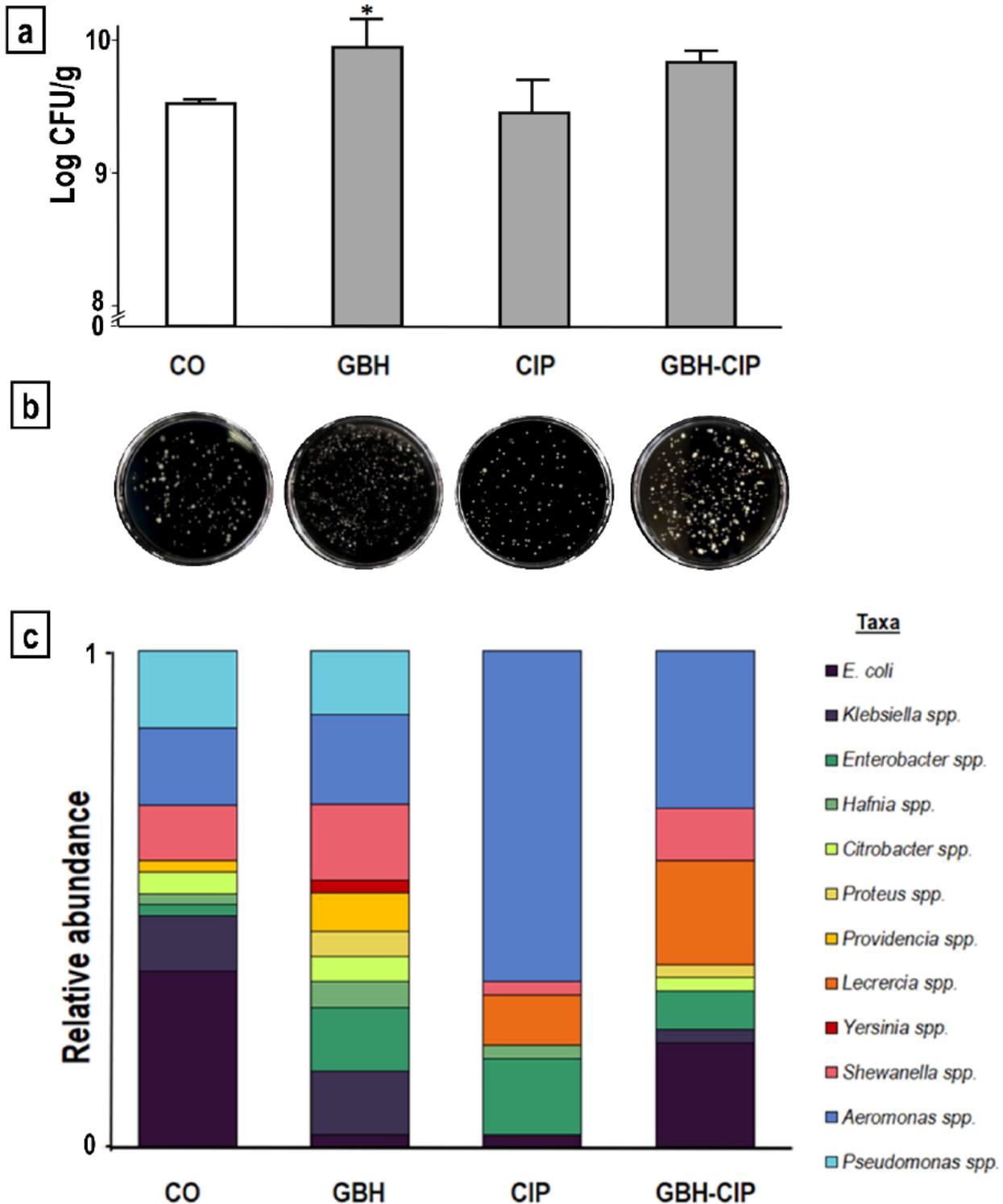


Figure 2

Comparison of the gut microbiota of tadpoles of *R. arenarum* exposed to glyphosate-based herbicide (GBH), ciprofloxacin (CIP) and their 50:50 % mixture (GBH-CIP), respect to control group (CO). a. Histogram of the plate count for CFUs (colony-forming units) /g of intestine, * significantly different from CO (Dunn post hoc test $p < 0.05$). b. Nutrient agar plates of pool samples from each treatment for CFUs count and sampling. c. Variation of taxa relative abundance on each treatment (cumulative frequencies of each genus over the total CFUs of each treatment, $n=60$)

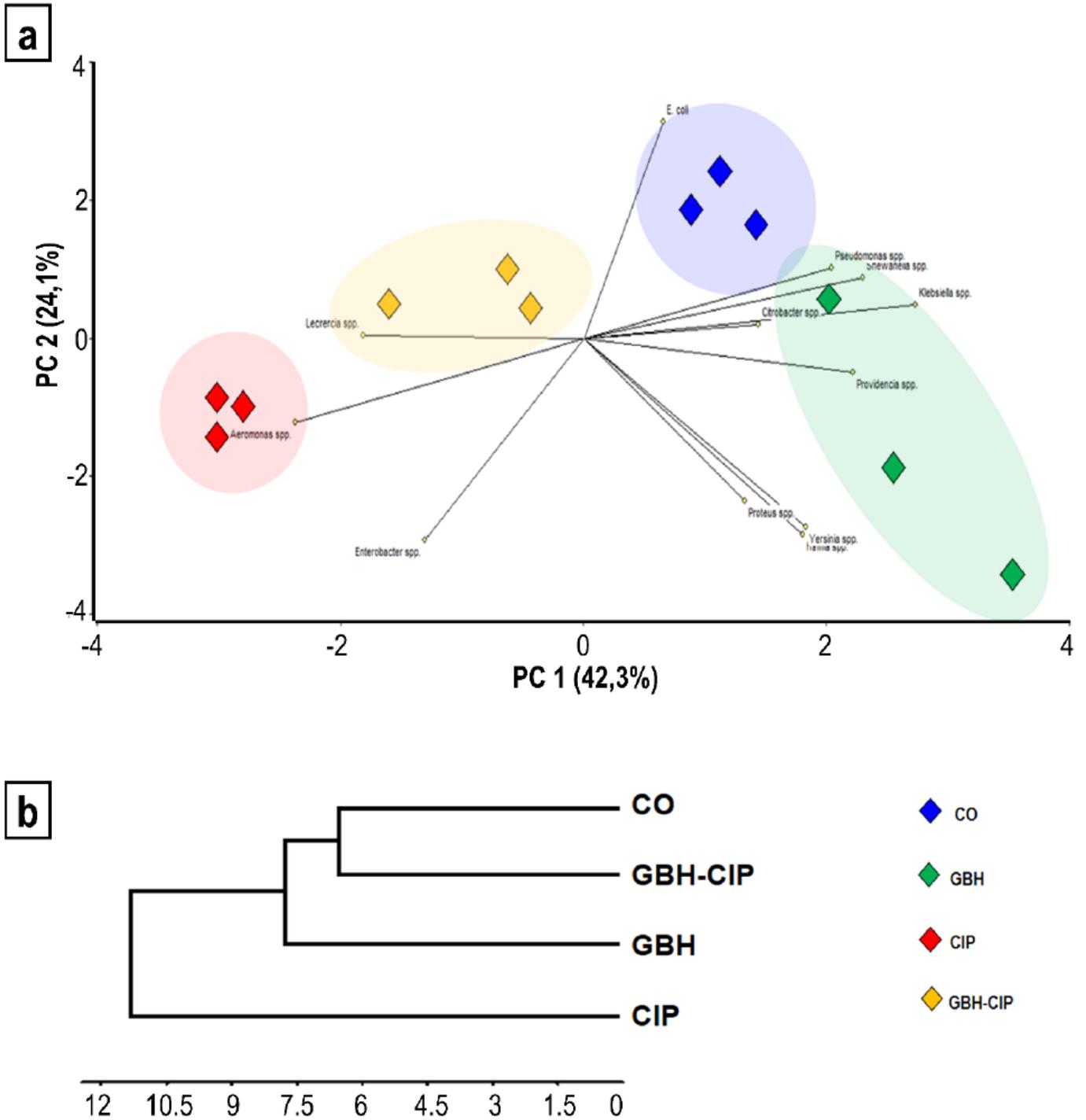


Figure 3

Beta diversity (variation of taxa composition) of gut microbial communities between treatments: control group (CO), glyphosate-based herbicide (GBH), ciprofloxacin (CIP) and their 50:50% mixture (GBH-CIP). a. The Principal Components Analysis biplot shows the patterns of separation for treatments based on different predominant taxa (genera); b. Hierarchical clustering (Unweighted pair-group method with arithmetic means) of treatments base on taxa composition (genus level).